Modulatory Effect of Mycobacterium Cell Wall Extract (Regessin) on Lymphocyte Blastogenic Activity and Macrophage Cytokine Gene Transcription in Swine

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Received 23 September 1996/Returned for modification 6 November 1996/Accepted 12 February 1997

Mycobacterium cell wall extract (MCWE) (Regessin) contains trehalose dimycolate and muramyl dipeptide, both of which have immunomodulatory properties. The goal of this study was to evaluate the effect of MCWE on the in vitro peripheral blood lymphocyte blastogenic activities to mitogens phytohemagglutinin (PHA) and concanavalin A (ConA) in 6- to 8-week-old piglets. The effect of MCWE on alveolar macrophage tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) gene transcription, as determined by a reverse transcription-PCR assay standardized with the endogenous glyceraldehyde-3-phosphate dehydrogenase gene, was also investigated. The results show enhanced blastogenic activities to mitogens PHA and ConA in MCWE-exposed cell cultures compared to those of control cell cultures. The enhanced blastogenic activity effect of MCWE was dose dependent. The cell background activity (spontaneous [3H]thymidine incorporation) of lymphocyte cultures was also significantly increased in the presence of MCWE, thereby demonstrating a lymphocyte mitogenic effect of MCWE. Cytokine gene transcription analysis showed that the TNF-α and IL-1β transcript levels in alveolar macrophage cell cultures stimulated with MCWE for 6 or 16 h were enhanced compared with those in control cell cultures. An enhancement of IL-1β mRNA levels in cell cultures stimulated for 16 h with MCWE, compared with those in control cell cultures, was also observed. The overall results demonstrate that MCWE can stimulate lymphocyte functional activity and cytokine mRNA expression in swine, thereby indicating its potential use as a clinical immunotherapeutic agent.

Immunocompetent cells, such as T and B lymphocytes and macrophages, play a major role in humoral and cellular immune responses to infectious agents and other antigens of various natures. A large number of immunostimulant agents are known to have the ability of potentiating nonspecific and antigen-specific immune responses through their modulatory effects on immune cells (9). Among these agents are bacterial products such as anaerobic corynebacteria (5) and mycobacteria (13). Bacillus Calmette-Guérin (BCG), a live strain of Mycobacterium bovis, has been used as a therapeutic agent for a number of medical conditions, including human bladder cancer (14, 24). However, its widespread use as a therapeutic agent has been limited by adverse reactions and the reluctance of physicians to use a live organism which may colonize and subsequently show infection in a patient (19, 28). It was discovered that the cell wall of this organism contains compounds, such as trehalose dimycolate (TDM) and muramyl dipeptide (MDP), subsequently shown to stimulate the immune system (13).

MDP represents the minimal structure of cell wall peptidoglycan, which is responsible for mycobacterium adjuvant activity in complete Freund’s adjuvant (3, 4, 10, 17, 25, 27, 36). MDP has also been shown to enhance nonspecific resistance to bacterial, fungal, and viral infections and cancer (10, 27). The primary target cell of MDP is the monocyte/macrophage (27). However, MDP has also been shown to positively modulate the activities of other immune cells, such as T- and B-lymphocyte blastogenic responses to mitogens, T-helper and T-cytotoxic antigen-specific responses, and B-lymphocyte-mediated antibody production, and to stimulate the production of various cytokines (3, 15, 17, 39). For instance, studies of murine and bovine species have shown that MDP stimulates tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, gamma interferon, and colony-stimulating factor production by macrophages (2, 10, 29). On the other hand, TDM represents the bioactive core factor found in bacteria, including Mycobacterium, Nocardia, and Rhodococcus spp. (21, 22). TDM has indeed been used as an immunomodulator in nonspecific immunotherapy against cancer and infectious diseases (6).

Mycobacterium cell wall extract (MCWE) is a commercial product (Regessin) prepared from nonpathogenic mycobacteria. It has been developed for its potential use in immunotherapy of numerous neoplastic conditions, targeting tumors which have historically been treated with BCG. MCWE is composed of a mixture of compounds, including MDP and TDM, which has been licensed to be used as an intratumor therapy of numerous neoplastic conditions, targeting tumors which have historically been treated with BCG. MCWE is composed of a mixture of compounds, including MDP and TDM, which has been licensed to be used as an intratumor cancer therapy for the treatment of canine mixed mammary tumors, canine adenocarcinoma, and equine sarcoid. We previously demonstrated an enhanced blastogenic activity of bovine lymphocytes to mitogens when they were exposed in vitro to MCWE (5). Here we extend our study of this product in swine to determine the immunomodulatory effect of MCWE on lymphocyte functional activity, as determined by an in vitro blood lymphocyte stimulation test with mitogens. The effect of MCWE on alveolar macrophage TNF alpha (TNF-α) and IL-1β gene transcription was also investigated by a reverse transcription-PCR (RT-PCR) assay standardized with the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

**MATERIALS AND METHODS**

**MCWE preparation.** The MCWE preparation (Regessin; 1 mg/ml) was generously provided by BIONICHE (London, Ontario, Canada).
Cells. The porcine peripheral blood mononuclear cells (PBMC) used in the blastogenic assay were separated by Ficoll–diazotize (specific gravity = 1.077) gradient centrifugation of sodium-citrate peripheral blood samples of individual 6- to 8-week-old specific-pathogen-free (SPF) pigs as previously described (34, 37). PBMC-derived adherent cell cultures (mainly monocytes) were obtained as previously described (37). The pig alveolar macrophage (PAM) cultures used for cytokine gene transcription were obtained from bronchoalveolar washes of individual 6- to 8-week-old SPF pigs as previously described (8, 37).

**Lymphocyte stimulation test.** In order to evaluate the effect of MCWE on peripheral blood lymphocyte functional activity, the in vitro lymphoproliferative activities to mitogens phytohemagglutinin (PHA) and concanavalin A (ConA) were assessed by a published procedure (5). PBMC were resuspended in RPMI 1640 medium containing 2 mM l-glutamine, 20 mM HEPES, 15% heat-inactivated fetal bovine serum (FBS), and antibiotics at a final concentration of 10^6 cells per ml. Two hundred microliters of PBMC suspension was placed in each well of a 96 flat-bottomed microtiter plate (Falcon, Lincoln Park, N.J.). Quadruplicate PBMC cultures received either 25 μl of cell culture medium without FBS or MCWE preparation shown to be free of endotoxin by the Limulus lysate test (4). MCWE was used at concentrations ranging from 1,000 to 3.1 μg per ml. Thereafter, each set of PBMC cultures received 25 μl of either cell culture medium without FBS (control cells) or PHA (Sigma Chemical Company, St. Louis, Mo.) or ConA (Pharmacia, Dorval, Quebec, Canada), with the last two used at suboptimal and optimal concentrations of 0.31 and 1.25 μg per cell culture. Lymphocyte cultures were then incubated for 2 days at 37°C in a 5% CO₂ atmosphere. All lymphocyte cultures were pulsed with 0.5 μCi of tritiated thymidine (specific activity, 6.7 Ci per mmol) (ICN Biomedicals, Inc., Sunnyvale, Calif.). The data were expressed in disintegrations per minute, and the retained radioactivity was then measured in a modulated LS1801; Beckman, Fullerton, Calif.). The means obtained for PBMC cultures in the absence of MCWE were 1,239 dpm for control cells (background), 236,959 and 109,878 dpm for cells stimulated with 1.25 and 0.31 μg of PHA, respectively, and 227,917 and 137,278 dpm for cells stimulated with 1.25 and 0.31 μg of ConA, respectively. The means obtained Δdpm values were 235,720 and 108,639 dpm for corresponding cell cultures stimulated with 1.25 and 0.31 μg of PHA, respectively, and 226,678 and 136,039 dpm for corresponding cell cultures stimulated with 1.25 and 0.31 μg of ConA, respectively (Fig. 1). The incorporation of MCWE in PBMC cultures induced a significant enhancement (P < 0.05 for 25 μg of MCWE per ml and P < 0.01 for 6.25 and 3.12 μg of MCWE per ml) of blastogenic cell activity in the presence of the optimal concentration (1.25 μg per well) of PHA, compared to that of control PHA-stimulated cell cultures without MCWE (Fig. 1A). No significant enhancement of the blastogenic response was obtained in cell cultures containing 12.5 μg of MCWE per ml. A significant enhancement of lymphoproliferative reactivity (P < 0.01 for 25 μg of MCWE per ml and P < 0.001 for 12.5, 6.25, and 3.12 μg of MCWE per ml per well) was also observed when PHA was used at a suboptimal concentration, 0.31 μg per cell culture. Similar results were obtained with PBMC from a different donor (data not shown).

**RESULTS**

In vitro nonspecific blastogenic response of porcine blood lymphocytes incubated in the presence of MCWE. The non-specific blastogenic activity results for porcine blood lymphocytes exposed in vitro to MCWE are presented in Fig. 1 and 2. The means obtained for PBMC cultures in the absence of MCWE were 1,239 dpm for control cells (background), 236,959 and 109,878 dpm for cells stimulated with 1.25 and 0.31 μg of PHA, respectively, and 227,917 and 137,278 dpm for cells stimulated with 1.25 and 0.31 μg of ConA, respectively. The means obtained Δdpm values were 235,720 and 108,639 dpm for corresponding cell cultures stimulated with 1.25 and 0.31 μg of PHA, respectively, and 226,678 and 136,039 dpm for corresponding cell cultures stimulated with 1.25 and 0.31 μg of ConA, respectively (Fig. 1). The incorporation of MCWE in PBMC cultures induced a significant enhancement (P < 0.05 for 25 μg of MCWE per ml and P < 0.01 for 6.25 and 3.12 μg of MCWE per ml) of blastogenic cell activity in the presence of the optimal concentration (1.25 μg per well) of PHA, compared to that of control PHA-stimulated cell cultures without MCWE (Fig. 1A). No significant enhancement of the blastogenic response was obtained in cell cultures containing 12.5 μg of MCWE per ml. A significant enhancement of lymphoproliferative reactivity (P < 0.01 for 25 μg of MCWE per ml and P < 0.001 for 12.5, 6.25, and 3.12 μg of MCWE per ml per well) was also observed when PHA was used at a suboptimal concentration, 0.31 μg per cell culture. Similar results were obtained with PBMC from a different donor (data not shown).

The lymphocyte blastogenic activity in cell cultures stimulated with 1.25 μg of ConA was significantly higher when MCWE was used at concentrations of 25 and 3.12 (P < 0.01) and 1.25 μg per ml (P < 0.01) per ml; it was also significantly higher in cell cultures stimulated with 0.31 μg of ConA when MCWE was used at a concentration of 12.5 to 3.12 μg per well (P < 0.001) (Fig. 1B). The overall results showed a significant diminution (P < 0.05 or 0.001) of the lymphocyte proliferative response to PHA or ConA in the presence of 50, 100, or 1,000 μg of MCWE per ml (Fig. 1). This diminution, which is comparable to that observed with mitogens for which the dependence of lymphocyte proliferation is typically bell shaped, does not appear to be due to a cell toxicity phenomenon, as determined by a trypan blue viability test with cells performed at the end of the incubation period and by the counts obtained in control cells which were not stimulated with PHA or ConA. No systematic experiments were conducted to explain such diminution. However, it is known that products like MDP may exert differential effects of cell stimulation or suppression, depending on the control of the PAMP procedure used for analyzing porcine IL-1 mRNA expression have previously been described (34, 37). Membranes were subjected to chemiluminescence analysis with a commercial digoxigenin luminescence detection kit (Boehringer Mannheim). Chemiluminescence-treated membranes were exposed to X-ray film (X-OMAT AR-5; Eastman Kodak Company, Rochester, N.Y.) for 2 to 15 min. The 25 μg of MCWE preparation used was found to be free of endotoxin by a published procedure (34, 37). Membranes were subjected to chemiluminescence analysis with a commercial digoxigenin luminescence detection kit (Boehringer Mannheim). Chemiluminescence-treated membranes were exposed to X-ray film (X-OMAT AR-5; Eastman Kodak Company, Rochester, N.Y.) for 2 to 15 min. The 25 μg of MCWE preparation used was found to be free of endotoxin by a published procedure (34, 37).
Clearly, further experiments are needed to determine the mechanism underlying this cell function inhibition.

The addition of MCWE at concentrations ranging from 25 to 3.12 μg/ml also induced a significant enhancement ($P < 0.001$) of spontaneous $[\text{H}]$thymidine incorporation in control cell cultures (cell background activity without mitogens PHA and ConA) (Fig. 2). These results suggest that MCWE has a lymphocyte mitogenic effect.

**Detection and analysis of cytokine gene transcription.** We also wished to evaluate the effect of MCWE on IL-1β and TNF-α gene expression in swine alveolar macrophage. To fulfill this objective, alveolar macrophage were cultured and exposed to MCWE or LPS (positive control) for 6 or 16 h. Then total cellular RNA was extracted and subjected to RT with a random hexamer primer as described previously (37). cDNA aliquots were coamplified by 30 (IL-1β) or 28 (TNF-α) PCR cycles with a primer pair specific to each cytokine together with the GAPDH primers in the same tube reaction (37).

As shown in Fig. 3A, lane 1, no TNF-α cDNA band (291 bp) could be detected in the nonstimulated control cell culture after 6 h on the ethidium bromide-stained agarose gel. In contrast, a TNF-α cDNA band derived from the cell culture exposed to MCWE for 6 h was slightly visible on the agarose gel and was nearly similar in intensity to that obtained in the LPS-stimulated positive control macrophage cell culture (Fig. 3A, lanes 2 and 3). This indicated enhanced TNF-α gene transcription when cells were exposed to MCWE. cDNA PCR fragments specific for the constitutive GAPDH gene obtained for control or MCWE- or LPS-stimulated cells were in the same range of intensity on the agarose gel. The agarose gel was then subjected to Southern hybridization with cDNA probes specific for the expected amplified sequences. As shown in Fig. 3B, specific hybridization signals were obtained with the TNF-α and GAPDH probes. It was apparent from the results obtained from the ethidium bromide-stained agarose gel and corresponding autoradiographs that the level of TNF-α gene transcription in cell cultures stimulated with MCWE or LPS for 6 h was increased compared with that in control, unstimu-
between control and MCWE-exposed cell cultures were observed in LPS-stimulated cells, though it was observed in cells exposed to MCWE for 16 h, though it was higher (Fig. 3C and D). Similar enhanced TNF-α gene transcription levels were also observed with MCWE-exposed alveolar macrophage cells from a different donor, as well as with MCWE-exposed blood-derived adherent cells from other donors (data not shown).

To generate semiquantitative data, the levels of TNF-α gene transcription in MCWE- and LPS-stimulated cell cultures relative to the endogenous GAPDH RNA level were determined and were compared with the cytokine/GAPDH ratio obtained in control cells at each cell posttreatment time. As shown in Fig. 3E, the TNF-α gene transcription levels after 6 and 16 h of stimulation with MCWE were enhanced compared to those of control cell cultures. However, these enhancements of TNF-α gene transcription levels were clearly smaller than those observed in LPS-stimulated cell cultures.

Porcine IL-1β gene transcription levels relative to the endogenous GAPDH mRNA levels after cell stimulation were also investigated. No differences in the levels of IL-1β mRNA between control and MCWE-exposed cell cultures were observed after 6 h of cell treatment (Fig. 4A and C). In contrast, an enhancement of IL-1β gene transcription was observed in cells exposed to MCWE for 16 h, though it was smaller than that obtained in LPS-stimulated cells, as shown by Southern blot hybridization autoradiographs of RT-PCR products and by IL-1β/GAPDH ratio determinations, compared to that of control, unstimulated cell cultures (Fig. 4B and C). As for TNF-α gene transcription analysis, similar enhanced IL-1β transcription levels were also observed with alveolar macrophage and blood-derived adherent cells from different donors (data not shown).

Detection of cytokine in cell culture supernatants. Soluble TNF-α was detected in LPS- and MCWE-stimulated blood adherent cell culture supernatants at 6 and 16 h after cell treatment. The cytokine levels in LPS-stimulated cell cultures were greater (120 versus 39 and 220 versus 100 pg/ml at 6 and 16 h, respectively). No cytokine was detected in control, unstimulated cells.

**DISCUSSION**

The stimulation of host defenses against infectious diseases and cancer is a much sought-after goal of immunotherapy. In view of this situation, it is not surprising that the immunopharmacology and clinical trials of new immunomodulatory drugs have developed rapidly during the last few decades. However, the potential use of these compounds in immunotherapy has often been hampered by the lack of knowledge of the basic mechanisms at the molecular level involved in the stimulation of the host immune system machinery. Therefore, the application of methods and use of molecular reagents to assess immune-cell functional activity as well as intrinsic activity, such as cytokine gene transcription, are of the utmost importance in elucidating certain aspects of the biology of these immunomodulatory agents and subsequently help to fulfill the regulatory requirements for their clinical application in animals and humans.

There is only one study of swine (9) in which mycobacterial compounds such as MDP and interphase material extracted from *Mycobacterium smegmatis* were reported to induce the enhancement of PAM cell viability, spreading and adherence on plastic surfaces, and IL-1 activity on appropriate indicator murine cells. In this study, the biology of MCWE, another mycobacterium-derived product, was assessed in porcine immune cells by its ability to modulate the blastogenic proliferative response to mitogens and the levels of cytokine (IL-1β and TNF-α) mRNA expression in alveolar macrophage. The results presented here indicate a significant enhancement of the blastogenic activities of MCWE-exposed mononuclear cells to PHA and ConA. The enhanced lymphoproliferative activity effect of MCWE was dose dependent. The blastogenic response was even greater in MCWE-exposed cell cultures that contained suboptimal concentrations of PHA and ConA, thereby demonstrating a potentiation effect of MCWE on lymphoproliferative activities to mitogens. A similar blastogenic potentiation effect of bovine or mouse lymphocytes to T-cell mitogens or a specific antigen has been reported for cells stimulated in vitro with MCWE (5) or mycobacterium derivatives such as MDP (12), which is an important component of MCWE. Moreover, MDP has also been shown to enhance the proliferation and effector functions of mouse cytotoxic lymphocytes activated by a suboptimal or optimal antigen concentration (12), as well as to potentiate antigen-specific T-helper-cell proliferation and activation when it was incorporated in a vaccine preparation against malaria in humans (17).

The results also show that MCWE induced a significant enhancement of the cell background activity (spontaneous [3H]thymidine incorporation), thereby demonstrating a mitogenic effect for porcine blood lymphocytes similar to that reported for bovine blood lymphocytes (5). Such results in the in vitro-enhanced background activity, as well as the enhanced blastogenic activity to mitogens PHA and ConA, could be explained by the fact that MDP, and presumably MCWE, has the macrophage as its primary target cell (27) and enhances IL-1 production in monocyte/macrophage-containing cultures (1, 10, 29). IL-1 has the ability, among others, to potentiate the proliferation and activation of resting and mitogen-exposed T and B lymphocytes by inducing the transcription, synthesis, and secretion of IL-2, as well as the expression of IL-2 receptors (11, 12). It is thus very likely that the macrophage production of IL-1 induced by both of these products would also enhance the blood lymphocyte blastogenic activities to mitogens PHA.
and ConA, which are primarily mitogenic for T cells in humans and many animal species.

Cytokines play a pivotal role in the activation of the host immune cascade. IL-1β, TNF-α, and IL-6 are cytokines that are mainly produced by monocytes/macrophages in response to stressful conditions and have a role in lymphocyte activation (23). Sequential cytokine induction by cells of the mononuclear phagocyte system has been observed in vitro and in vivo after endotoxin (LPS) stimulation (29). MDP has also been reported to induce TNF-α, IL-1, and/or IL-6 mRNA accumulation and protein secretion in mouse and bovine macrophages (1, 10, 29) and in human peripheral blood monocyte cultures (32). TNF-α activity has also been found in serum and lung tissue samples from TDM-inoculated mice (35).

In this study, experiments were conducted to determine the TNF-α and IL-1β gene transcription levels in PAM and blood-derived monocytes exposed in vitro to MDP- and TDM-containing MCWE. Enhancements of porcine IL-1β and TNF-α mRNA transcription levels in alveolar macrophage were observed after 6 and/or 16 h of stimulation with MCWE. Such enhanced levels of cytokine gene transcription have also been reported for endotoxin- and MDP-stimulated immune cells from humans and other animal species (7, 16, 18, 20, 26, 31). Similar IL-1β and TNF-α transcription level enhancements were also obtained with porcine blood-derived adherent mononuclear cells incubated with MCWE. Detectable levels of soluble TNF-α, as well as IL-6 biological activity, as determined by a proliferation assay with IL-6-dependent B9 cells (37) (data not shown), were found in the supernatant fluids of blood-derived adherent cell cultures incubated with MCWE. Therefore,
IL-1β transcription level enhancement in blood-derived adherent cell cultures, and presumably the secretion of the protein, may very well account, as mentioned above, for the potentiating effect of MCWE on the blastogenic activities in control and PHA- and ConA-stimulated porcine blood lymphocyte cultures observed in this study. Experiments (for instance, blocking IL-1 activity with porcine IL-1-specific antibodies) are needed to confirm this interpretation, should porcine-specific reagents become available.

The results reported here have shown that MCWE stimulated the porcine blood lymphocyte proliferative activities to mitogens and IL-1β and TNF-α gene transcription in alveolar macrophage and blood monocytes in swine. The potency of the effect of MCWE on the stimulation of immune-cell activity, reported here for the first time for swine and previously for the bovine (5), argues for further research to determine the feasibility of clinical applications in animals and humans to enhance natural resistance to tumors and infectious agents and for treatment of immunodeficiency states. Experiments to determine in vivo the effect of MCWE on the kinetics of gene expression and serum secretion of cytokines in swine are currently planned. This might be of some interest for nonspecific treatment of certain infections in swine, for instance, the porcine reproductive and respiratory syndrome infection, which is caused by a virus for which the target cell is the alveolar macrophage (8). Finally, it should be noted that MCWE is already incorporated as an adjuvant in certain animal vaccines and that human clinical study trials are in progress in Canada to evaluate MCWE in the treatment of carcinoma in situ of the bladder and localized carcinoma of the prostate.

ACKNOWLEDGMENTS

This work was supported by operating grant no. 3254 from the Conseil des Recherches en Pêche et Agro-Alimentaire du Québec (CORPAQ). S.-A. Vézina is supported by a graduate student fellowship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (Fonds FCAR) of the province of Québec. D. Archambault is the holder of a research scholarship from the Fonds de la Recherche en Santé du Québec (FRSQ).

We are grateful to Robert Bilodeau and Serge Dea for providing SPF piglets and alveolar macrophage, respectively. We also thank Dominique Roberge for technical assistance and BIONICHE for providing the MCWE preparation. We also thank Carole Villeneuve for secretarial work and Martin-Robert Blanchette for computer-generated images.

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